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13. ABSTRACT *(Maximum 200 Words)*

This study involves the detection of prostatic epithelial cells in the seminal fluid. Relatively normal cells are identified by Cytokeratin 8/18. Up-regulated, or cancerous cells, are identified by means of PSMA staining. Increased prostate specific membrane antigen is associated with a poor prognosis. Data is being collected to differentiate the seminal fluid values in a ratio between the cytokeratin cells and the PSMA positive cells. At the present time, these clearly distinguish between BPH patients and prostate cancer patients, with prostatitis patients being intermediate. As more and additional patients are followed for repeat studies, and additional patients are seen from our control vasectomy clinic, we will define this test to its ultimate possibilities. The obtaining of fresh samples and the elimination of viable sperm have been two of our key progress notes at this particular time.

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INTRODUCTION: The American Cancer Society (ACS) suggests that patients with elevated levels of prostate specific antigen (PSA) (4-10 ng/mL) undergo a biopsy and/or transrectal ultrasound (TRUS). This allows the clinician to evaluate the condition of the prostate, as PSA levels do not differentiate between benign prostatic hyperplasia (BPH), prostatitis, or prostate cancer. These procedures, although invasive and at times unpleasant for the patient, are necessary in diagnosing and planning the treatment strategy for the patient. Over 1.5 million prostate biopsies are performed annually based on ACS data, with only two out of five biopsies being positive for prostate cancer. Our research purpose is to minimize the need for invasive procedures by identifying and characterizing prostate cells that can be found in seminal fluid. Our research shows that prostatic intraepithelial neoplasia (PIN), benign and prostate cancer cells can be found in seminal fluid [1]. Very large tumors providing many cells do not present a major diagnostic issue. Our goal is to identify smaller tumors producing significant seminal fluid cancer cells that can lead to a positive biopsy.

OBJECTIVE 1: Define the parameters associated with the collection and preparation of semen samples and optimize procedures to maximize cellular quality.

In the past year we found the amount of sperm in the seminal fluid sample is a major area of concern, as it interferes with flow cytometry analysis. The sperm removal procedure we developed takes advantage of the fact that viable sperm are motile, and tend to swim against the direction of gravity. The freshness of the seminal fluid is crucial to sample preparation and must be processed for analysis within one hour of ejaculation. The sperm must be viable (visibly motile) for optimal removal.

The seminal fluid sample is liquefied at 37° C for one hour and then diluted in 10 mL of 37°C media. The diluted sample is slowly centrifuged to lightly "pack" the cells at the bottom of a rounded tube. The supernatant containing mostly sperm is removed and another 10 mL of 37°C media is added to the pellet while the sample sits at 37°C for one hour, allowing the remaining sperm to swim up from the pellet. Analysis by flow cytometry shows this separation method is more successful than separation gradients, slow centrifugation without adding fresh media to the pellet, and gravitational separation (allowing the diluted sample to sit at 37°C for at least one hour).

In samples containing very high concentrations of sperm cells ($>10^7$/mL), the pellet is resuspended in another 10 mL of warm media and the procedure is repeated to further remove sperm from the sample. In samples that have visibly low concentrations of sperm ($<10^5$/mL) no attempts are made to remove the sperm, as interference is minimal and the potential loss of round cells is detrimental to the study.

After the sperm removal process the resulting pellet is fixed in 50% ethanol fixative solution and stored until analysis.

Another contributing factor to the quality of the specimen is the sample volume from the donor. Because most of our donors are elderly men, the volumes of the samples are usually less than 2 mL. With volumes less than 0.5 mL it is difficult to acquire any cells for analysis, so these samples are not currently included in the study. With sample volumes of 3.0 mL or greater the concentration of sperm is too high to remove enough sperm and leave enough round cells for a valid analysis. A sample volume of this size is rare, however, but is useful in defining the sample parameters.

The following parameters have been set for optimization of cellular quality from the seminal fluid sample: 1) Freshness; the sample must be no more than 1 hour post-ejaculation, or the sperm must visibly be at least 90% viable, upon start of the procedure. 2) Volume; the sample must have a volume of 0.5 mL or greater, and preferably less than 3 mL, or have a sperm concentration of 5×10^6/mL or less, post-sperm removal. With these parameters in place we have increased the detectable DNA-cycling cell population from the previous 0.1% of the round cell population to approximately 1%.

OBJECTIVE 2: Continually perform semen cytology analysis on samples from: volunteers with no evidence of prostate cancer, patients with BPH or prostatitis, and patients with biopsy proven prostate cancer. In addition to a "screening" population we will follow multiple prostate cancer patients through the initial course of treatment and will evaluate the semen cytological data against the clinical conditions of each patient group.

We have performed semen cytology analysis on samples from: 90 volunteers with no evidence of prostate cancer, 52 patients with benign prostatic hyperplasia (BPH) or prostatitis, and 22 patients with biopsy proven prostate cancer. In addition we performed multiple timepoint analyses, over a period of 18 months, on a group of five prostate cancer patients who chose no treatment for their cancer. We will be increasing the number of patients in our multiple timepoint analysis group to at least 15 patients in the coming year.

To date our additional analysis includes 79 patients diagnosed with either BPH or prostatitis, or biopsy proven prostate cancer. We compare our patient samples to seminal fluid

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samples collected from normal, healthy vasectomized males under the age of forty, donated by The Vasectomy Clinic (Seattle, WA).

The results of these analyses agree with our initial findings and the ability to distinguish prostate cancer patients from normal donors (Fig. 1). This is to say that PSMA:cytokeratin ratios for the patients were consistently elevated, at an average near 0.5, whereas the ratio for the normal donor samples averaged near 0.1.

We are increasing the number of study patients with prostate cancer and suspected prostate cancer from our brachytherapy program, both pre-treatment and post-treatment. Our Foundation conducts two free prostate screening clinics attended by 1,500 males annually. All are screened for PSA levels and followed up by digital rectal exam. All patients with suspected prostate cancer are being offered the chance to participate in this study. With larger numbers of individuals participating we hope to increase our ability to identify trends in different stages of prostate disease.

OBJECTIVE 3: Identify new markers and incorporate them into our current system. Reevaluate against multiple patient samples with the purpose of developing panels of antibodies.

We have shown that prostate cells can be found and characterized in seminal fluid using the PSMA antibody 7E11 and the anti-cytokeratin 8/18 antibody CAM 5.2. We developed a three-color staining scheme, which incorporates these two antibodies, and a DNA stain that separates the DNA-cycling epithelial cells from the haploid sperm cells [1].

We have researched the incorporation of an anti-PSA antibody (399) as a more accurate means of characterizing prostate cells in seminal fluid. Our research shows that using an anti-PSA antibody does not provide sufficient separation of the cells for characterization (Fig. 2). It is our conclusion that there is too much extracellular PSA for a four-color scheme including PSA (CAM 5.2, 7E11, 399, To-Pro 3) to be beneficial.

Our research on the incorporation of other marker antibodies for a four color analysis is ongoing.

KEY RESEARCH ACCOMPLISHMENTS

- Optimization of cellular quality; by researching and selecting parameters for sample acquisition and processing we are now assured that we are receiving consistent, viable samples.
- Development of a process to remove most sperm cells from seminal fluid, which has reduced sperm interference during flow cytometry analysis.
- The discovery of high concentrations of extracellular PSA, preventing its use as a cytological marker in this study.
- The PSMA:cytokeratin ratio tends to distinguish PIN and prostate cancer from BPH and patients with no evidence of disease. The ratios for prostatitis have been intermediate thus far.

REPORTABLE OUTCOMES

- We are seeking local Foundation support for the purchase of a Laser Scanning Cytometer from Compucyte Corporation for the quoted price of $172,000. We are confident that this technology will increase the sensitivity of this assay.

CONCLUSIONS

Our research shows that prostate cancer cells can be detected in the seminal fluid of patients with known prostatic disease. Our goal is to be able to use this research to develop a diagnostic test for men with questionable disease, and to provide an alternative or follow-up measure prior to or after a biopsy.

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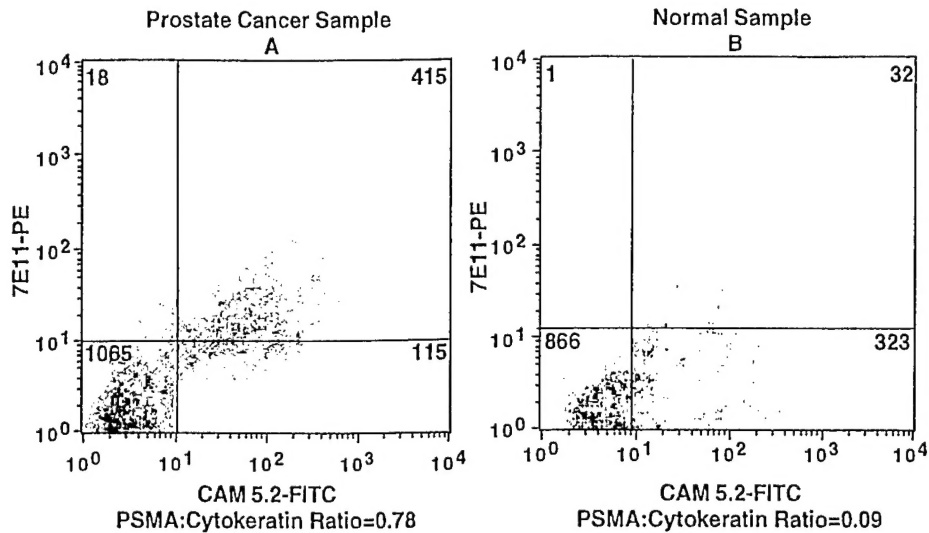
Prostate Cancer Sample
A

7E11-PE

18 · · · 415

1065 · · · 115

CAM 5.2-FITC
PSMA:Cytokeratin Ratio=0.78

Normal Sample
B

7E11-PE

1 · · · 32

866 · · · 323

CAM 5.2-FITC
PSMA:Cytokeratin Ratio=0.09

Figure 1
Representative dot plots of (**A**) a prostate cancer patient, CaP (T2a), and (**B**) a patient without any evidence of disease (NED). These graphs were generated from the DNA-cycling populations as determined by DNA pulse width and area graphs. Quadrants are based on the staining of the control cell lines LNCaP (CAM 5.2$^+$, 7Ell $^+$) and PC-3 (CAM 5.2$^+$, 7E11$^-$), run in conjunction with the patient samples. The CaP sample demonstrates the 7E11-posritive staining of the cytokeratin population and the relative lack of staining in the normal (NED) patient.
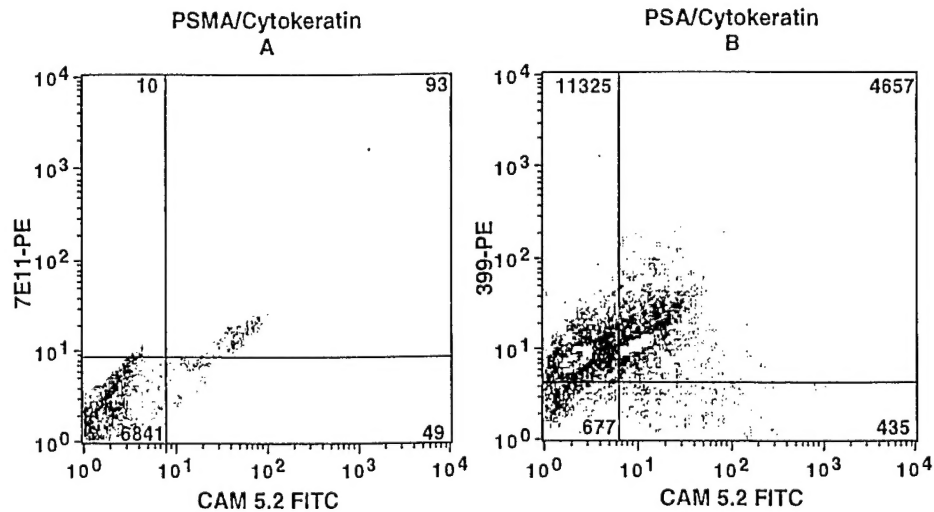

PSMA/Cytokeratin
A

7E11-PE

10 · · · 93

6841 · · · 49

CAM 5.2 FITC

PSA/Cytokeratin
B

399-PE

11325 · · · 4657

677 · · · 435

CAM 5.2 FITC

Figure 2
Dot plots representing a single cancer patient (T2a) showing the comparative staining between PSMA and PSA. These graphs were generated from the DNA-cycling populations as determined by DNA pulse width and area graphs. Quadrants are based on the staining of the control cell lines LNCaP (CAM 5.2$^+$, 7E11$^+$, PSA$^+$) and PNCF 007 (CAM 5.2$^-$, 7E11$^-$, PSA$^-$), run in conjunction with the patient samples. **A:** PSMA/cytokeratin staining shows that only cells positive for CAM 5.2 will stain positive for PSMA. **B:** PSA/cytokeratin staining shows that PSA staining is indiscriminate for CAM-positive or negative cells.